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以 *Pichia pastoris* 進行重組融合塵蟎過敏原 Der p 1 and 2 之表現

Expression of recombinant Der p 1 and 2 fusion allergen by *Pichia pastoris*

蔡旻憬
Min-Ching Tsai

指導教授：李昆達 博士
Advisor: Kung-Ta Lee, Ph.D.

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口試委員會審定書

以 *Pichia pastoris* 進行重組融合塵蟎過敏原 Der p 1 and 2 之發酵生產

Expression of recombinant Der p 1 and 2 fusion allergen by *Pichia pastoris*

本論文係蔡旻憬君（R95B47102）在國立臺灣大學微生物與生化學研究所完成之碩士學位論文，於民國九十七年六月十九日承下列考試委員審查通過及口試及格，特此證明

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Abstract

To efficiently generate recombinant allergens of *Dermatophagoides pteronyssinus* for allergen-specific immunotherapy such as the oral tolerance, we linked its major allergens, Der p 1 and Der p 2, into a fusion protein and expressed it via the system of *Pichia pastoris*. pPICZαA vector was chosen to construct a protein-secretable system because of the existence of α-factor signal sequence in front of the N-terminal of the fusion protein. Sequence of the linker linking the two proteins was designed to form a α-helix conformation after translated into amino acid sequence. The α-helix was expected to prevent the fusion protein from folding interference of the two recombinant allergens. After pPICZαA-Der p 1-linker-Der p 2 plasmid was constructed and transformed into the *Pichia pastoris* X33 (wild type) via electroporation, we selected a Mut^+ strain which showed the highest productivity with an ELISA specific to Der p 2. The productivity of the fusion allergen in Hinton's flask was 35.5 μg/mL after 72 h of methanol induction. We also analyzed the samples with Western blotting specific to Der p 2. The Western blotting showed some smear and higher molecular weight bands, which were coincided with our prediction that the existence of an N-glycosylation within Der p 1 amino acid sequence would leads to glycosylation. A high cell density culture in the Bioflo110 fermentor was achieved with

427 mg/mL wet biomass, and 203 mg/L fusion allergen production. According to the Western blotting and ELISA results, it concludes that we successfully constructed a fusion allergen expression strain of *P. pastoris* and produced the fusion allergen via a high cell density culture.

Key words: fusion allergen, α-helix, α-factor signal sequence, *Pichia pastoris,* N-glycosylation.

中文摘要

為了有效地提供歐洲塵蟎的重組過敏蛋白進行過敏原專一性的口服免疫治療，我們將第一類 (Der p 1) 及第二類 (Der p 2) 這兩個最主要的歐洲塵蟎過敏原蛋白連接成融合蛋白，並透過 *Pichia pastoris* 加以表現與生產。因 pPICZαA 質體在融合蛋白的 N 端前方具有 α-factor 外泌訊息序列，因此我們選取此質體來建構外泌蛋白的表現載體。連接兩個蛋白間的 linker 序列是設計成在轉譯成胺基酸序列後會形成 α-螺旋，α-螺旋被認為可以避免融合蛋白質在折疊成正常構型時互相干擾。成功的建構了 pPICZαA-Der p 1-linker-Der p 2 質體後，此質體經電轉形進入 *Pichia pastoris* X33 (野生型) 中。我們利用針對 Der p 2 之三明治酵素連結免疫分析法篩選出一株表現量最高的 Mut^+ 菌株。此融合過敏原蛋白在 Hinton 氏搖瓶中培養與甲醇誘導 72 小時後產量為 35.5 μg/mL。同時，我們利用針對 Der p 2 的西方墨點法分析樣品；西方墨點法的結果顯示出模糊拖尾且在較高的分子量位置的蛋白質條帶，這與我們所預測由於 Der p 1 的胺基酸序列中有一個 N-糖基化的位置會造成糖基化之結果相符合。在 Bioflo110 發酵槽中進行細胞高密度培養，其細胞濕重可達 427 mg/mL ，目標融合過敏原蛋白的產量則為 203 mg/L。三明治酵素連結免疫分析法及西方墨點法的結果顯示，我們成功地在 *Pichia pastoris* 系統建構了一株融合過敏原蛋白的表現菌株並可透過細胞高密度培養生產此融合過敏原蛋白。

關鍵字：融合過敏原蛋白，α-螺旋，α-訊息序列，畢赤酵母，N-醣基化

Abbreviation

BSA	Bovine serum albumin
cDNA	Complementary DNA
Der p 1	*Dermatophagoides pteronyssinus* 1
Der p 2	*Dermatophagoides pteronyssinus* 2
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
kDa	Kilodaltons
LB	Luria-Bertani
OD	Optical density
PCR	Polymerase chain reaction
rpm	Rotation per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrlamide gel eletrophoresis
TMB	3,3',5,5'-tetramethylbenzidine
vvm	Volume of air per volum of medium per minute

專有名詞 中英文對照表

Dermatophagoides pteronyssinus 1	歐洲室內塵蟎過敏原 1
Dermatophagoides pteronyssinus 2	歐洲室內塵蟎過敏原 2
Electrophoresis	電穿孔法
HRP	辣根過氧化物酶
Immunoprophylactic	免疫預防
Monoclonal antibody	單株抗體
PCR	聚合酶連鎖反應
Plasmid	質體
Polyclonal antibody	多株抗體
Primer	引子
Promoter	啟動子
Restriction enzyme (endonucleoase)	限制酶
Sandwich-ELISA	三明治酵素免疫分析法
Superthem polymerase	Superthem 聚合酶
Western blotting	西方墨漬法

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Chapter I

Introduction

1.1 House dust mite allergens

Hypersensitivity to house dust mite (*Dermatophagoides* sp.) allergens is one of the most common allergic reactions. Estimates suggest that as many as 10% of the general population and 90% of individuals suffering from allergic asthma are sensitive to house dust mites. The severity of the problem is on the rise, with at least 45% of young people with asthma showing sensitivity (Derewenda et al., 2002). *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* are the major source of indoor allergens associated with various allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis. In Taiwan, *Dermatophagoides pteronyssinus* is the predominant house dust mite (Kuo et al., 1999).

The examination of house dust mite extracts has indicated that over 20 different proteins can induce IgE antibody in patients allergic to the house dust mite. Table 1 summarizes the biochemical and IgE-binding characterization of the known house dust mite allergens. Group 1 (Der f 1 and Der p 1) and group 2 (Der f 2 and Der p 2) allergens derived from house dust mites are considered major allergens based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract (Thomas et al., 2002). Out of these reasons, study of major house dust mite allergenic proteins, such as Der p 1 and Der p 2 is very important for

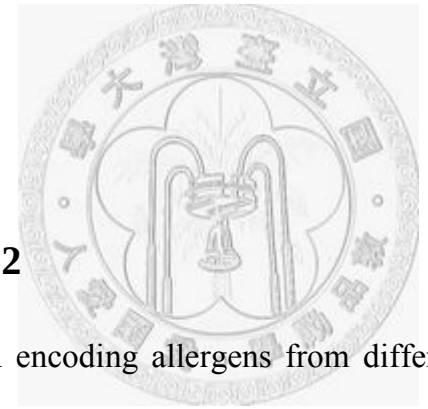
immunotherapy research (LinWan-Yi, 2007).

1.1.1 Allergen Der p 1

Group 1 allergen produced by the *Dermatophagoides pteronyssinus* (Der p 1) has been described as an aeroallergen with a molecular mass of 27 kDa, carried on mite feces. It is a glycoprotein with cysteine protease activity and the structure of Der p 1 was determined by comparative modeling with papain actinidin and papaya protease of the cysteine protease family (de Halleux et al., 2006). The protease activity is able to cleave human CD25 and CD23. This activity enhances total and specifc IgE production in mice immunized with proteolytically active Der p 1. Protease activity of Der p 1 has also been reported to increase the permeability of the human respiratory epithelium (Gough et al., 1999; Takai et al., 2002; Adam et al., 2006).

Although cDNA clones encoding group 1 allergens from *Dermatophagoides pteronyssinus* (Der p 1) have been available for many years, attempts to produce biologically active recombinant versions of these allergens have been only moderately successful, most likely because these molecules have complex molecular features. These features include a 19-residue signal peptide, an 80-residue

pro-enzyme sequence, a 222- to 223-residue mature protein, a potential N-linked glycosylation site, and three cysteine residue pairs assumed to be involved in disulfide bridges (Best et al., 2000). During the latest decade, *Pichia pastoris*-drived recombinant Der p 1 was successful constructed and expressed. After their efforts in the reveal of structure and protease activity of Der p 1, the biological efficiency of *Pichia pastoris*-drived recombinant Der p 1was almost the same as the native Der p 1 (Sakata et al., 2004; Takai et al., 2005b; de Halleux et al., 2006; Ogawa et al., 2008).



1.1.2 Allergen Der p 2

To date, the cDNA encoding allergens from different protein groups of the house dust mite have been cloned and sequence. The Group 2 allergens are considered to be major allergens because 80-90% of mite-allergic individuals produce humoral and cellular responses to these allergens (Heymann et al., 1989). The group 2 allergen of *Dermatophagoides* sp. induce high titers of IgE (van der Zee et al., 1988; Trombone et al., 2002) and Th2 cytokines in 80% of allergic patients (Hales et al., 2000; Hales et al., 2002). The group 2 allergens were first characterized as 14,000-18,000 MW allergens with a high IgE-binding activity

(Thomas et al., 2002). The house dust mite allergen Der p 2 is a major allergen and 80-90% of patients who react to mite extract, react specifically to this protein or its homologues (Heymann et al., 1989; Sporik et al., 1992; Platts-Mills et al., 1997). Der p 2 is thermostable that it can be preserved in room temperature for a month (Cain et al., 1998).

Der p 2 from *D. pteronyssinus*is is a single-domain protein with an immunoglobulin fold composed of 129-amino-acid residues (Takai et al., 2005a). The molecular mass of Der p 2 is 14 kDa and it is devoid of *N*-glycosylation sites. Sequence analysis shows that house dust mite group 2 allergens (Der p 2, Der f 2, Lep d 2, etc.) exhibit a 35% identity to the human epididymal epithelial cell secreted protein (HE1), which is known to bind cholesterol with high affinity (Naureckiene et al., 2000). It suggests that house dust mite group 2 allergens may play a role in mite reproduction (Thomas and Chua, 1995)

The solution structure of Der p 2 determined by NMR methods has been reported (Mueller et al., 1997). The crystal structure shows that Der p 2 possesses an immunoglobulin-liked internal cavity, which is created by two anti-parallel β-sheets. The cavity is lined with hydrophobic and aromatic amino acid residues, so the ligand molecules must be hydrophobic. This architecture suggests strongly that the Der p 2

molecule has evolved to bind lipid-like molecules (Derewenda et al., 2002). In this context, it is interesting to note that there are reports in the literature of lipid-binding proteins eliciting an allergic response (Mantyjarvi et al., 2000; Asero et al., 2001; Furmonaviciene and Shakib, 2001).

1.2 Immunotherapy of oral tolerance and edible vaccine

1.2.1 House dust mite extract used in immunotherapy

House dust mite allergen crude extracts are currently used throughout the world for both diagnostic and specific immunotherapy procedures (Taketomi et al., 2006). House dust mite extracts are made from an aqueous extraction of variable mixture of whole mites, nymphs, faecal pellets, eggs and spent culture media (Thomas et al., 2002). Accordingly, some patients may be misdiagnosed and some may respond poorly to specific immunotherapy because they are treated with mixed allergen extracts that contain an inappropriate level of the relevant allergens (Thomas and Smith, 1998). Thus, skin prick test positive to a certain mite crude extract will indicate that the patient is sensitized to mite allergens, but without identifying the specific allergenic proteins. Mite extracts contain at least 19 well characterized allergens, and mite sensitized patients could have differential IgE reactivity to the

major allergens.

1.2.2 Oral tolerance

Oral administration of antigen proteins would be presented by intestinal lymphoid tissue, which can induce a group of antigen-specific regulatory T-cells to suppress antigen-specific immune response. This is called “Oral tolerance” (Ho, 2002). It was first described in 1911 when Wells fed hen's egg proteins to guinea pigs and found them resistant to anaphylaxis when challenged. In 1946, Chase fed guinea pigs the contact sensitizing agent dichloronitrobenzene (DCNB) and observed that animals had decreased skin reactivity to DCNB (Weiner, 1994). High dose of antigen administration to many autoimmune murine models had been well studied and some of them were extremely successful in alleviating disease severity (Weiner, 1997; Benson et al., 1999; Wardrop and Whitacre, 1999)

Based on the long history of oral tolerance and the apparent safety of the approach, human trials have been initiated in multiple sclerosis, rheumatoid arthritis, and uveitis (Trentham et al., 1993). The phenomenon of oral tolerance has also been observed in humans fed and immunized with keyhole limpet hemocyanin (50 mg) (Husby et al., 1995). Because of the supply of natural protein antigen is limited,

several animal models of low dose tolerance (0.2 to 1 mg) have been investigated. The natural allergen cases (Wiedermann et al., 1999; Sato et al., 2001) successfully reduced the specific IgE titer or Th2 cytokine production and showing some tolerance effects in BALB/c mice models.

1.2.3 Edible vaccines

The oral tolerance immunotherapy has it potential to evolve into an edible vaccine. In order to economic production of immunoprophylactics, it has resulted in the concept of generating them in recombinant form through protein expression systems or transgenic foods at relatively low cost (Pinzon-Charry, 2007). Lots of work has been successful developed via recombinant proteins expressed in fruits, crops and other foods as the materials for oral tolerance research. A rice-based edible vaccine expressing multiple T cell epitopes of Japanese cedar pollen was successfully developed (Takagi et al., 2005b; Takagi et al., 2005a; Hiroi and Takaiwa, 2006; Takagi et al., 2006). It induced the oral tolerance for inhibition of Th2-mediated IgE responses (Takagi et al., 2005b; Takagi et al., 2005a; Hiroi and Takaiwa, 2006; Takagi et al., 2006; Takaiwa, 2007; Yang et al., 2007; Yang et al., 2008). Till now, including expression of recombinant SARS-CoV spike protein, chicken anemia virus proteins, *Helicobacter*

pylori heat-shock protein A in transgenic tobacco plant (Li et al., 2006; Zhang et al., 2006a; Lacorte et al., 2007); Cholera toxin B protein, Norwalk virus capsid protein expressed in transgenic tomato fruit (Zhang et al., 2006c; Jiang et al., 2007; Santi et al., 2008) were reported.

Two basic strategies have been employed: the expression of full recombinant antigens or antibodies, or the presentation of small antigen epitopes in transgenic foods (Lal et al., 2007). Both approaches have resulted in the production of biologically active immunoprophylactics that have been shown in animal studies to protect from the development of disease symptoms (Hansen and Kawashima, 2000). An especially interesting feature of producing vaccines in edible plant parts is the potential to use the transgenic plant as a vehicle for oral delivery (Streatfield, 2006). We can see these edible vaccines as a needle-free vaccine delivery (Giudice and Campbell, 2006). Immunisation without needles could have medical and technical advantages as well as being less traumatic for children (Tonks, 2007).

1.3 Expression of recombinant Der p 1 and Der p 2 in different hosts

For using in immunotherapy, skin-pick testing or protein structural studies,

mass production of allergen proteins is therefore needed. Because of the high price of the natural allergy proteins from dust mite extract, efficient strategies for production of recombinant proteins are gaining more and more important. The cDNA of Der p 1 was isolated in 1988 and expressed in *E. coli* (Thomas et al., 1988); in addition, the cDNA coding for Der p 2 was isolated by Chua, et al. in 1990 and initially expressed in *E. coli* as a glutathione- S-transferase fusion protein (GST-Der p 2). After that, different expression host for expression of recombinant allergy proteins include prokaryotic and eukaryotic systems were developed. There are some examples of various recombinant allergen expressed in different systems (Table 2).

1.3.1 Expression of recombinant Der p 1

1.3.1.1 Expression of rDer p 1 in *E. coli*

In 1988, the first approach to cloning and expression of DNA coding for the mjor house dust mite allergen Der p 1 in *Escherichia coli* was achieved. In briefly, the mRNA of *Dermatophagoides pteronyssinus* was isolated and then synthesis the cDNA library. Anti-Der p 1 antiserum was used to screen *Der p 1* gene. The Der p 1 gene was confirmed and the predicted amino acid sequence of Der p 1 was determined. The *Der p*

1 gene was then sent into *E. coli* Y1089 and used lambda-gt11 as the expression vector. However, at that time the expression of Der p 1 was unsuccessful because the soluble protein in lysate was low and the purification was not easy (Thomas et al., 1988).

1.3.1.2 Expression of rDer p 1 in *Drosophila* cells

The cloning and expression of a secreted form of proDer p 1 was conducted in insect cells. The purified recombinant allergen was compared to Der p 1 isolated from whole mite culture extracts, in term of biochemical, enzymatical and immunological properties. Almost the same enzymatic activity was observed between the natural Der p 1 and the rDer p 1. The ELISA results showed that antigenic properties are preserved in the recombinant protein and that pro-Der p 1 maturation is necessary for immune recognition with specific IgE or monoclonal antibodies. The pro-Der p 1 could express at the high level of 20 mg/L within culture medium in 24 wells plates, but the level of production decreased drastic when expression was performed in a large scale.(Jacquet et al., 2000)

1.3.1.3 Expression of rDer p 1 in mammalian cells

Expression of recombinant house dust mite allergen proDer p 1 with synthetic gene

codon in mammalian cells was successful: expression of recombinant ProDer p 1 was secreted by CHO cells (Massaer et al., 2001). As the low GC content of the Pro-Der p 1 gene (38%) may impair expression in mammalian cells, a synthetic Pro-Der p 1 gene was generated in which the codon usage was optimized for expression in CHO cells. The results showed that the optimization of the codon usage is essential for Pro-Der p 1 expression at a high level in CHO cells. The recombinant Pro-Der p 1 was shown to be less allergenic than the natural Der p 1 and the productivity was 34 μg/mL.

1.3.1.4 Expression of rDer p 1 in *Pichia pastoris*

Expression of recombinant Der p 1 in *P. pastoris* was successful and the maturation of Der p 1 from pro-Der p 1 was discussed in several researches (Jacquet et al., 2002; Takai et al., 2002; van Oort et al., 2002). Der p 1 was characterizes as a cysteine protease (Takai et al., 2005b). The proteolytic activity of a purified recombinant Der p1 was thought to be with commitment of sensitization toward IgE and IgG responses (Gough et al., 1999; Kikuchi et al., 2006). Glycosylation was observed when using *P. pastoris* as an expression host. Site-direct mutation was also used for rDer p 1 to substitute the glycosylation site within the amino acid sequence (van Oort et al., 2004). The highest productivity of Der p 1 in *P. pastoris* was 150 mg/L.

1.3.2 Expression of recombinant Der p 2

1.3.2.1 Expression of rDer p 2 in *E. coli*

The production of recombinant allergens by microorganisms has the advantages including easily handling, saving time and cost compared to the purification of dust mite extract. *Escherichia coli* is the host organism frequently used for the production of recombinant proteins. Der p 2 has been easy to produce as a recombinant allergen in *E. coli* (Chua et al., 1991; Tame et al., 1996; Mueller et al., 1997). Although the recombinant protein must be re-natured by an dilution refolding method, high level expression of rDer p 2 protein from *E. coli* has been reported (Mueller et al., 1997). Nevertheless, the rDer p 2 protein are accumulated as insoluble inclusion bodies within *E. coli* cells (Takai et al., 2005a). Also, the contamination of endotoxin for animal model test and the incorrect conformation folding are the disadvantages of producing recombinant Der p 2 in an *E. coli* expression system.

1.3.2.2 Expression of rDer p 2 in yeasts

Saccharomyces cerevisiae has been the major yeast expression system for recombinant proteins production since early 1980s (Hitzeman et al., 1981). rDer p 2 can be produced and secreted from *S. cerevisiae* in a form essentially

indistinguishable from the natural allergen. This recombinant allergen is ideal for structural studies or immunological research or the study of mutants and the modified allergens (Hakkaart et al., 1998). Oral feeding with low dose of rDer p 2 generated from yeast would decrease Der p 2-specific IgE or IgG1 titers following immunization (Ho, 2002). In comparison to *E. coli*, yeast-based expression systems offer the advantages of eukaryotic folding machinery, the absence of endotoxin, and a higher yield of the recombinant protein (Krebitz et al., 2000). However, yeast expression system has some disadvantages, such as hyperglycosylation of recombinant protein or the foreign proteins are often commonly degraded.

1.3.2.3 Expression of recombinant Der p 2 in plants

Except *E. coli* and yeast, the recombinant Der p 2 had been expressed in transgenic tobacco plant, its suspension cells, and transgenic potato plant for development of low-cost edible oral vaccine (Ho, 2002; Wang et al., 2003). Compared with *E. coli* and the yeast, oral tolerance induced with rDer p 2 from suspension cells of transgenic tobacco had the profoundest effect to alleviate allergic airway inflammation (Ho, 2002). The advantages of expressing foreign proteins in transgenic plants and their suspension cells was mentioned above.

1.4 Fusion proteins and the protein linker

Protein fusion technology is an indispensable tool for biochemical researches. Recombinant fusion proteins have been adopted as a means to increase the expression of soluble proteins, and to facilitate protein purification such as the Histag technology. Furthermore, immunoassays using chimeras by fusion with an antibody fragment or antibody binding domain had been applied; using enzymes or green fluorescent protein in the selection and production of antibodies and the engineering of bi-functional enzymes were also reported (Graciet et al., 2003; Wu et al., 2003).

A fusion protein must involve in the linking of two proteins by a peptide linker. To choose a proper linker sequence is particularly important. A flexible linker or a fixed linker such as α-helix linker is the choice for linkers design. Researches has shown that a fixed α-helix linker design could be a proper linker peptide because of it is effective to separate two domains to be folded correctly and with their nature functions (Arai et al., 2001).

1.5 *Pichia pastoris*

Pichia pastoris was developed into an expression system by scientists at Salk

Institute Biotechnology/Industry Associates (SIBIA) for high-level expression of recombinant proteins (Higgins et al., 1998). As an eukaryotic expression host, its cellular physiology is similar to animal cell therefore should express Der p protein structures more similar to the native fold (Martinez-Donato et al., 2006; Zhang et al., 2006b). *P. pastoris* could perform N-glycosylation as well to ensure posttranslational modification (Koutz et al., 1989; Abdulaev et al., 1997; Bretthauer and Castellino, 1999). *P. pastoris* has a greater surface/volume ratio and the cells could grow at a faster rate and have a greater scale-up ability. The expression of foreign protein under AOX1 promoter was inducible by addition of methanol (Koutz et al., 1989). Therefore expression of proteins or enzymes that were toxic to the host cells could be performed at a high cell density culture (Gellissen, 2000; Macauley-Patrick et al., 2005; Chang et al., 2006; Martinez-Donato et al., 2006; Lannoo et al., 2007).

Pichia pastoris was available as a kit from Ivitrogen corp. and several special expression strains have been constructed. X33 is the natural wild type of *Pichia pastoris*, other mutant were manipulated from this strain. GS115 was a histidine deficient strain. KM71H, SMD1168 and SMD1163 were protease deficient strains. Mutants may have better productivity under specialized and designed programs of

fermentation for each recombinant protein (Khatri and Hoffmann, 2006; Zeder-Lutz et al., 2006). There are a series of vectors could be used when designing the expression vector. pPIC9, pPICZ, pPICZα..etc,. Z means Zeocin resistance. Zeocin resistance can be useful when selecting *E. coli* or *Pichia pastoris* transformed strains. Furthermore, different concentrate of Zeocin can be useful for selecting a higher copy number strain after *Pichia pastoris* transformation in advance of the ELISA for recombinant protein quantification. Alpha means α-factor, an α-factor secret signal can be design in front of expression protein to ease the purification steps (Daly and Hearn, 2005).

1.6 Aim of this study

The *Pichia pastoris* which can produce rDer p 2 was kindly provided by prof. B.L. Chiang. The rDer p 2 can be secreted during high cell density culture in the total synthetic medium. The secretion of rDer p 2 eased the recovery process from cultured medium. We also constructed the ELISA and Western blotting systems that specific to Der p 2 for quantification and qualitative analysis (LinWan-Yi, 2007).

In this study, we want to construct a recombinant fusion allergen gene encoding *Der p 1* and *Der p 2* by an α-helix linker in pPICZαA vector, and then transformed into

Pichia pastoris. The linker was designed to be adequate not long for efficient expression and not short to lose the function. We hope the α-helix linker could separate the two allergens with a proper distance. If the α-helix linker was useful and fused Der p 2 and Der p 1 could fold independently, the Der p 2 ELISA and Western blotting system will show the signal during conduction. We can also observe the glycosylation of Der p 1 during Western blotting. The α-factor signal was included in the plasmid construction for protein secretion. Histaq was designed to be located after the sequence of the fusion allergen. This vector was transformed into *Pichia pastoris* X33 for recombinant protein expression. A high productivity strain was chosen and expressed in Hinton's flask and then scale up cultured in a fermentor. This is the first report for fusion major house dust mite production. We hope that this recombinant fusion allergen could be a good material for oral tolerance research and a pretest for development of the edible vaccine.

Chapter II

Materials and Methods

2.1 Microorganisms and vectors

Escherichia coli JM109 (Yeastern Biotech, Taipei, Taiwan) was chosen for vector preservation. *Pichia pastoris* X33 (Invitrogen, Sandiego, USA) were chosen as the yeast expression hosts. yT&A vector (Yeastern Biotech, Taipei, Taiwan) was used as a gene preservation vector for constructing the fusion allergen expression. The expression vector for *P. pastoris* was pPICZαA (Invitrogen, Sandiego, USA). *Der p 1 and Der p 2* gene respectively inserted in pGEX vector (named pGEX-Der p 1 and pGEX-Der p 2) was kindly provided by Professor Chiang (Graduate Institute of Clinical Medicine, Medicinal College, NTU) as a gift. The synthesized linker nucleotide sequence and primers were purchased from Blossom biotechnologies, Taipei, Taiwan.

2.2 Construction of plasmids and the *P. pastoris* expression host

pPICZαA vector contained an α-factor signal sequence which allowed the recombinant fusion allergen protein downstream of the signal sequence to be secreted in *P. pastoris* system. The complete sequence of pPICZαA is available for downloading from Invitrogen company World Wide Web site (www.invitrogen.com). A map of pPICZαA vector and the multiple cloning sites are shown in Fig 1. At the

C-terminus of the α-factor secretion signal peptide, there is a Kex2 signal cleavage site right before the last four amino acids (Glu-Ala-Glu-Ala), which could be cleavaged by *KEX2* gene product for separating the α-factor signal peptide and the fusion allergen. Fusion allergen gene was attached immediately behind Kex2 signal sequence cleavage site by the polymerase chain reaction (PCR) methods between *Eco*RI and *Xba*I restriction sites. Table 3 shows the linker sequence and the primers used. Figure 1 is the schematic representation of Der p 1 - Der p 2 fusion protein. We constructed fusion allergen gene in yT&A vector, and then send it into pPICZαA for transformation.

2.2.1 Construction of the fusion allergen gene in yT＆A vector and then pPICZαA vector

PCR which used pEcoRI-Der p 1 combined with pDer p 1-XhoI as the primer, and pGEX-*Der p 1* as the template constructed a full-length mature *Der p 1* gene. PCR which used pSacII-Der p 2 combined with pDer p 2-XbaI as the primer, and pGEX-*Der p 2* as the template constructed a full-length of *Der p 2* gene. PCR which used pXhoI-linker combined with plinker-SacII as the primer, and the synthesized single strand linker sequence as the template constructing a 78 base pair of double strand linker sequence. All PCR introduced restriction site as the primer name they

used followed by a 6 base pair sequence to the 5'-end and the 3'-end as the underlined letters in Table 3.

The PCR mixture and the thermalcycling conditions were as following:

PCR mixture:	
10X PCR buffer (Qiagen)	5 μL
5X Q solution (Qiagen)	10 μL
4 mM dNTP	3.75 μL
10 μM forward primer	2.5 μL
10 μM reverse primer	2.5 μL
Template	2 μL
H_2O	22.45 μL
Superthem DNA polymerase (0.2 U)	2 μL
	Total volume: 50 μL

Thermalcycling conditions:

94°C 5 min	
94°C 30 sec	30 cycles
55°C 30 sec	
72°C 30 sec	

The PCR product was confirmed by electrophoresis using 1.2% agarose gel under 100 voltage in 1XTAE buffer. Product of the appropriate size was recovered by NucleoSpin kit (Qiagen, Germany) with the protocol suggested in the user manual (Vogelstein and Gillespie, 1979). After cutting out the region of the appropriate size with a razer, this portion of agarose gel was mixed with 600 μL of NT1 buffer and

incubated in 50°C for 10 min. During this step, the agarose gel completely dissolved and the solution was transferred into an extraction column adapting to a collection tube. Spin down the column under 8,000xg for 1 min and then discard the flow through. Add 600 μL of NT3 buffer into the extraction column and spin down under 12,000xg for 1 min. Add 200 μL of NT3 buffer and spin down under 12,000xg for 1 min. Transfer the extraction column to a sterilized microcentrifuge tube. Drip 10 μL of NE buffer on the membrane of the extraction column and let it stay for 1 min, spin down under 12,000xg for 1 min, then repeat this step again. The PCR product of the appropriate size was collected in the microcentrifuge tube.

Der p 1, *Der p 2* and the linker gene coding sequence with tailored end sequence possessing restriction enzyme sites obtained from PCR is then immediately transferred into a yT&A cloning vector and preserved in *E. coli* strain JM109. The ligation reaction of tailored *Der p 1, Der p 2* and linker gene into yT&A cloning vector is as following:

Ligation reaction of TA cloning:	
yT&A cloning vector	2 μL
buffer A	1 μL
buffer B	1 μL
tailored *Der p 1, Der p 2* and the linker gene or other genes should be preserved during the manipulation of genes	5 μL
yT&A T4 DNA ligase	1 μL

	Total volume: 10 μL

The ligation mixture was kept at 4°C overnight and then added into 100 μL half-thawed *E. coli* JM109 competent cells. Heat shock at 42°C for 45 sec, the transformants were grown on LBA plates and selected by blue/white screening (Sambrook and Russell, 2001). Prior to spreading the transformants, 100 μL 100mM IPTG and 20 μl 50mg/mL X-gal was added on the surface of the plates. Colonies grown were first checked by colony PCR to see whether the appropriate base pair size was presented and the sequences were confirmed by sending transformed colonies to Tri-I Biotech (Taipei, Taiwan) for sequencing service.

With the correct sequence confirmed, we started to construct the fusion allergen gene. Because of the restriction of linker size that can't be seen within electrophoresis, we directly ligate the *Der p 2* gene and linker sequence after processed with *Sac*II endonucleases right after PCR of *Der p 2* gene and the linker sequences. The reaction mixture for restriction enzyme digestion and ligation were as follows and the remaining reactions for restriction enzyme reactions were as follows with modification of enzymes and buffers:

Restriction enzyme reaction:	
10X reaction buffer	2 μL
Substrate DNA	2 μL

*Sac*II	2 μL
H_2O	14 μL
	Total volume: 20 μL

After ligation, linker-*Der p 2* gene was further ligated into a yT&A vector. For the full-length of *Der p 1*-linker-*Der p 2* gene construction, *Der p 1* and linker–*Der p 2* genes preserved in different yT&A vectors were digested with *Xho*I and *Xba*I endonucleases and then electrophoresis to separate the products. We cut the yT&A-*Der p 1* and linker-*Der p 2* from the gel and recovered them with NucleoSpin kit. yT&A-*Der p 1* and linker-*Der p 2* genes were ligated into yT&A -*Der p 1*-linker-*Der p 2*. The full-length of *Der p 1*-linker-*Der p 2* gene with proper restriction enzyme sites were constructed in yT&A.

Because of the ligation of *Der p 2* and the linker was conducted directly after digestion of the PCR products, unconfirmed *Der p 2* and linker genes were further switched for the previously confirmed genes preserved in yT&A vector. With the correct sequences, yT&A vector containing tailored *Der p 1*-linker-*Der p 2* gene was then processed with *Xho*I and *Xba*I endonucleases. Empty pPICZαA vector was processed with the corresponding restriction enzymes as well. Then, the *Der p 1*-linker-*Der p 2* was ligated into a pPICZαA within *Xho*I and *Xba*I restriction enzyme sites. The shuttle vector pPICZαA-*Der p 1*-linker-*Der p 2* was then transformed into *E.*

coli JM109 and sending for sequencing. With the correct sequence confirmed, the shuttle vector containing the right sequence with proper reading frame was preserved in *E. coli* JM109 and selected by LSLBZ plate (Low salt LB plate with 25 μg/mL of Zeocin.)

2.2.2 Construction of *P. pastoris* expression host

The pPICZαA vector did not contain yeast replication origin, thus, transformants could be isolated only recombination did occur between the plasmid and the host genome. *P. pastoris* X33 was chosen as the expression hosts for expressing fusion allergen. X33 represents the wild-type of *P. pastoris*. Expression vectors were transformed into the host by electroporation according to the protocol provided by Invitrogen with a few improvements (Becker and Guarente, 1991; Chang, 1992). *P. pastoris* strain was incubated in 5 mL YPD medium at 30°C, 150 rpm shaking overnight, and then transferred into 100 mL YPD medium, incubated overnight again at 30°C, 150 rpm shaking until OD_{600} reached 1.3~1.5. The overnight cultured cells were collected by centrifuging at 2,000xg for 5 min at 4°C. Then the cells were washed 2 times with ice-cold H_2O, once with 50 mL and the second time with 25 mL, cells were collected by centrifuging at 2000xg for 5 min at 4°C. The cells were then washed

with ice-cold 1M sorbitol, once with 2 mL and the second time resuspended in 100 μL. These cells were kept on ice and used that day.

The expression vector pPICZαA-*Der p 1*-linker-*Der p 2* was linearized for electroporation. For linearization, *Sac*I endonuclease was used under the following reaction mixtures:

Linearization reaction:	
10X L buffer	2 μL
pPICZαA-*Der p 1*-linker-*Der p 2*	2 μL
H_2O	15 μL
*Sac*I	1 μL
	Total volume: 20 μL

Reaction mixtures were kept at 37°C for 90 min. The linearized expression vectors were obtained and checked by electrophoresis using 1.2% agarose gel and purified by Nucleospin kit (Qiagen, Germany) with the protocol described above.

The electroporation mixture was setup by mixing 80 μL of cells prepared above and 2 μL of linearized expression vector previously mentioned. Transferred the mixture to an ice-cold 0.2 cm electroporation cuvette and stayed on ice for 5 min. Then the mixture was pulse under 1.5 KV, 25 μF, 200 ohm for 5 msec, and then quickly added 1 mL ice-cold 1M sorbitol. Gently resuspended the cells, and the mixture was transferred into a sterilized glass tube and the tube was incubated at 30°C without shaking for 90 min. Spread every 300 μL of incubation mixture on a YPDSZ plate,

incubated the plates at 30°C until colony forms (5~6 days). Colonies were streaked again on YPDZ plates, and incubated at 30°C until colony formed. Colony PCR were taken during further selection to confirm the fusion gene. Primer used were 5' AOX combined with 3'AOX to confirm fusion gene were insert into the right position; pEcoRI-Der p 1 combined with plinker-SacII or pXhoI-linker combined with pDer p 2-XbaI to confirm the fused gene.

2.3 Selection of a proper expression strain

2.3.1 Selection of a Mut^+ expression strain

Recombination of plasmid and *Pichia pastoris* genome may result in the destruction of AOX1 gene because of the substitution of AOX1 gene by the heterologous gene. Destruction of AOX1 gene leads to that metabolites alcohol poorly is called a Mut^s strain, and the preservation of AOX1 gene called a Mut^+ strain that sustains a good ability in alcohol metabolite.

We used MD (Dextrose) and MM (Methonal) plates which with different carbon source to select the Mut^+ strains. Bigger colonies from the YPDSZ plates were selected to streak on the MM and MD plate, respectively. Colonies showed high growth rate on MM plate were picked for further selection.

2.3.2 Selection of a strain with high copy-number

During gene recombination, multiple gene-insertion events at a single locus in a cell do occur spontaneously but with a low, detectable frequency--between 1 and 10% of all selected Zeocin resistant transformants (Daly and Hearn, 2005). With higher copy number, colonies could grow on higher concentration of Zeocin plate. We used a series of concentration of Zeocin on YPDZ plates (from 50 μg/mL to 1000 μg/mL) to select strains with higher copy number.

After Mut^+ selection, selected colonies were streaked on YPD plate and cultivated at 30^oC until colonies formed. Colonies were picked and suspensed in 10 μL of YPD medium respectively. One μL suspension of each colony was dropped on YPDZ plates with different concentrate of Zeocin. Colonies grown on 1000 μg/mL Zeocin were picked and streaked on YPD plates for the next selection.

2.3.3 ELISA selection for higher productivity in small scale cultures

After Zeocin selection, strains were cultured in tubes for further selection for strains with high expression efficiency. Colonies with high copy numbers were picked from the YPD plate and cultured in 2 mL of YPD medium for 24 hours. After 24

hours, we added 1 μL of 100% methanol to achieve 0.5% of methanol induction within the YPD medium for further 24 h cultures. Finally, supernatant was collected by centrifuging at 14,000xg for 10 min at 4°C. Supernatant was conducted the sandwich-ELISA (as described below) for selection of high productivity strains.

2.4 Cultures in the Hinton's flasks

After the above selections, the two strains (strain 6 and strain 33) expected with high productivity and a random chosen strain (strain 10) for comparison were cultured in Hinton's flasks to investigate the strategy we took was right. First, three strains were respectively cultured in 25 mL of BMG medium which contained glucose as the sole carbon source at 30°C, 150 rpm shaking overnight. The overnight cultured cells were collected by centrifugation at 2,000xg for 10 min at 4°C and further resuspensed in 100 mL of BMM medium which contained methanol as the sole carbon source for expression induction. Induction was carried out for 24 hours and added 0.5 mL of methanol per 24 h for 72 hours totally continuous induction. Samples were collected per 24 h and supernatant were collected by centrifugation at 14,000xg for 10 min at 4°C. Supernatant were assayed by ELISA and the high productivity strain was chosen.

2.5 Cultures in fermentors

After the highest productivity strain was selected, we cultured it to produce the fusion allergen in Bioflo 110 fermentor via high cell density cultures. Culture conditions within the fermentor were set at 30^{o}C, 1000 rpm and pH-stat at 5. DO probe was used to monitor the oxygen tension and the air-flow set at 1vvm and gradually increased to 3 vvm. when the cultures achieved a high cell density. The colony picked from YPD plate was cultured in 100 mL BMG medium at 30^{o}C, 150 rpm overnight as a seed culture. One hundred mL of broth was inoculated into 2.5 L FBSM medium and grew for 24 h to deplete the glycerol as the 1st stage, and the wet biomass reached to 100 g/L. During the continued 6 hours, 250 mL of 50% glycerol was fed at a rate of 18.15 ml/h/liter for the initial fermentation volume, and achieved 200 g/L wet biomass at this stage. At the 30th h, methanol induction was started at a feeding rate of 3.6 ml/h/liter (to the initial fermentation volume). The methanol feeding rate rose to 7.2 ml/h/liter when the dissolved oxygen stopped fluctuation and rose to 20%. Finally, the methanol induction continued until the wet biomass stop rising. Samples were collected every 3 h during the first 36 h and every 12 h for the remaining culture. Supernatant was collected by centrifugation at 12,000xg for 10 min at 4^{o}C and the wet biomass was measured at the same time. The fusion allergen in supernatant was

measured by sandwich-ELISA and the total soluble protein was determined by the Bradford method (Bradford, 1976). Western blotting was also conducted to detect the fusion allergen.

2.6 Quantification of total soluble protein

Total soluble protein was determined by the Bradford method according to the Bio-Rad-Microassay Procedure (Bio-Rad, CA, USA). A standard curve was produced with known concentrations of BSA (Sigma-Aldrich, MO, USA).

2.7 Preparation of recombinant Der p 2 protein as the standard in ELISA and the Western blotting

P. pastoris transformed with pPICZαA-*Der p 2*, kindly provided from Prof. B.L. Chuang as a gift, was used to produce the rDer p 2 as the standard. After cultured in a Bioflo 110 fermentor including 4 days of methanol induction, 3 litters of supernatant were recovered. The supernatant contained mainly the 15 kDa molecular weight of rDer p 2 and some minor 70K molecular weight of protease. After filtered with a 30 kDa Amicon Ultra membrane (Millipore, USA), the protease was depleted. We further used 5 kDa Amicon Ultra membrane for desalting and to concentrate the rDer p 2. To make

sure the rDer p 2 had been correctly folded, the concentrate was treated with refolding reagent (8 M urea) (Takai et al., 2005a).

2.8 Sandwich-ELISA

We used sandwich-ELISA specific for quantification of rDer p 2. First, 96-well flat-bottomed polystyrene microplates (TPP® Techno Plastic Products AG, Trasadingen, Switzerland) were coated with 100 μL of coating buffer (Carbonate-bicarbonate, pH 9.6) which contained 1/100 (v/v) diluted anti-Der p 2 monoclonal antibody. After plates blocked with gelating-NET (0.25% gelatin, 0.15 M NaCl, 5 mM EDTA, 0.05% Tween in 50 mM Tris-HCl, pH 8.0), samples (100 μL) were respectively added to the wells and incubated at 37°C for 1 hour. And then, anti-Der p 2 polyclonal antibody from rabbit at a 1:1000 dilution was added to each well and incubated at 37°C for 1 h. After the wells washed by phosphate buffer saline with 0.05% Tween-20 (PBST), 100 μL of Goat anti-Rabbit IgG (H+L) HRP conjugated affinity purified antibody (Chemicon, CA, USA) at a 1:3000 dilution was added to each well and incubated at 37°C for 1 h. After the wells were washed, the 1-Step™ Ultra TMB-ELISA substrate (Pierce, IL, USA) was added and incubated for 20 min to 30 min until the blue color appeared. The Der p 2 concentrations were determined by comparison to a standard curve of rDer p 2 from

yeast.

2.9 Western blotting

To conduct the experiments of Western blotting of Der p 2, the supernatant was mixed with 5x loading dye and was boiling at 100°C for 8 min, first the samples and the rDerp 2 produced by yeasts as a standard were run on SDS-polyacrylamide gels, respectively (running gel: 15%, stacking gel: 5%) and accompanied with LandMarker Mid range Prestained Protein Marker (Mbiotech, Seoul, Korea). The electrophoresis was performed under 150 V for 40 min. The gels were blotted onto Hybond-C Extra membrane (Amersham, London, UK) at 85 V for 50 min followed by blocking with 5% skim milk (Becton, Dickinson and Company, MD, USA) overnight. The membranes were incubated in blocking buffer with the anti-Der p 2 polyclonal antibody as the 1st antibody. Then the membranes were incubated with Goat anti-Rabbit IgG (H+L) HRP conjugated affinity purified antibody as the 2nd antibody. All the incubation process was performed at 50 rpm rotary shaker (Firstek, Middlesex, UK) at room temperature for 1 h. Antibody binding was detected after incubation with chemifluorescence reagents (PerkinElmer Life Science, Inc., MA, USA) and detected by UVP BioImaging System (AutoChem^{TM} System, CA, USA).

Chapter III

Results

3.1 Construction of the fusion allergen gene

Because the linker sequence was only 78 base pairs (Table 1), it is difficult to see the PCR product with DNA gel electrophoresis during gene cloning, so we directly ligated the PCR products of linker sequence and *Der p 2* gene with *Sac*II site, and then the *Der p 1* gene was added to the front with *Xho*I site for a full-length of our design. The possibility of mutation during PCR is high, but with a strategy "constructed the full-length first, and then replaced the wrong ones second", we successfully constructed the vector containing *Der p 1* linked with *Der p 2* without any incorrect nucleotide.

Compared with the data in NCBI website, the full length of *Der p 1* gene is 907 bp and *Der p 2* is 591 bp. In figure 2, the *Der p 1* we cloned was 684 bp and *Der p 2* was 395 bp. This is that Der p 1 belongs to a cysteine protease with a pre-sequence and a pro-sequence in the front of its N-termini. The pre-sequence was thought to be used to bring the protease to the right position within house dust mite. Mature Der p 1 with cysteine protease activity is forming after the pro-sequence was deposited. Der p 2 also contains a pro-sequence although the function remains unknown. Based on the above information, we directly cloned the mature domain of Der p 1 and Der p 2, respectively, for efficient production of the recombinant allergens. Fig 3 showed the sequencing result of constructed gene in pPICZαA.

3.2 Selection of a higher productivity strain

Figure 4 shows the results of Zeocin selection. After 4 days of culture, these strains expressed different tolerance abilities at different concentration of Zeocin (500μg/mL and 1000μg/mL). Strains showed higher tolerance to Zeocin were kept on YPD plate and then transferred to 2 mL of YPD medium for further methanol induction.

After Zeocin selection, colony PCR were taken to confirm the fusion gene were insert into the right position. Figure 5-1 showed the result of colony PCR using 5'AOX and 3'AOX as primer, the insert DNA (1146 bp) plus AOX gene (588 bp) were 1734 bp. Strain 31 were deleted for no result in colony PCR. Two more colony PCR used pEcoRI-Der p 1 combined with plinker-SacII or pXhoI-linker combined with pDer p 2-XbaI were taken to confirm the gene were correctly fused (Fig 5-2 and Fig 5-3).

Figure 7 shows the ELISA results (for fusion allergen) in small scale methanol inductions (Figure 6: Standard curve of Der p 2 ELISA). We found strain 6 and strain 33 both were higher expression strains. Therefore we chose strain 6, 33 and a randomly selected (lower productivity) strain 10 for further comparisons. The supernatants were concentrated 10-fold and were analyzed by Western blotting and ELISA.

Figure 8 shows the ELISA results of strain 6, 10 and 33 cultured for different time of methanol induction. The fusion allergen yield increased with a longer induction time,

and strain 33 was apparently a higher yield strain. Our results showed strain 33 grew better than strain 10, but similar to strain 6. It was consistent to the results of Zeocin selections. It demonstrated that Zeocin selection was efficient for selection of a high yield strain. The ELISA system specific to Der p 2 was used for quantification of the fusion protein, and the yield was further calibrated by a ratio of 45k/15k (molecular weight of fusion allergen protein / molecular of rDer p 2 standard).

3.3 Western blotting of products of strain 33

Figure 9 shows the results of Western blotting. rDer p 2 standard showed a sharp band at the lower site in the page however, the target samples showed a smeare phenomenon at the higher site. Compared with the primary membrane of this Western blotting which loaded with a prestained marker, rDer p 2 standard was at the right position around 15 kDa (date not shown). Recombinant fusion allergen was predicted with a 45kDa molecular, however, it showed smear and shifted to the position during 65 to 90 kDa. It may be caused by the N-glycosylation of *P. pastoris*, because of a N-glycosylation could be happened at the matured amino acid of Der p 1 (N52). It had been known that one site of N-glycosylation for a protein could result in smear at the sites of higher molecular weight (Yasuhara et al., 2001; Takai et al., 2002; van Oort et

al., 2002). The appearance of smear could be detected in our Western blotting system also suggests that Der p 1 had been expressed and fused with Der p 2.

3.4 Cultures of strain 33 in Hinton's flask and in a fermentor

Before cultured in a fermentor for high cell density cultures, strain 33 was cultured and then treated 72 h of methanol induction for fusion allergen expression in Hinton's flasks (Fig.10). The fusion allergen in medium achieved 35.5 μg/mL (n=3), and the wet biomass dropped after 48 h of induction. It suggests that the carbon source were depleted at the stage. Comparison to the flask cultures contained BMG (M) medium, which only produced 50 g/L in wet biomass.

Figure 11 shows the production results of strain 33 cultured in a fermentor. During the initial 24 h, the wet biomass accumulated slowly and reached to 99.6 g/L. During 24 h to 30 h, 250 mL of 50% glycerol was fed and the wet biomass rapidly increased to 227.9 g/L. Methanol induction started at the 30th h and the wet biomass raised slowly before 60th h because *P. pastoris* needed time to adapt to the new carbon source (methanol feeding rate was increased from 9 ml/h to 18 mL/h at this stage). During the stage of *P. pastoris* adapted to methanol (60~96th h), the wet biomass increased with a higher growth rate and achieved 400 g/L in wet biomass (methanol

feeding rate was kept at 18 mL/h at this stage). In the last stage of methanol feeding, the wet biomass accumulated slowly and finally achieved 427 g/L in wet biomass at 132th h (methanol feeding rate was gradually adjusted from 18 mL/h to 12 mL/h at this stage for not to accumulate that would be toxic to cells). The OD_{600} also showed a similar trend compared to the accumulation of wet biomass. In the stage of initial log phase (18~30th h) and the stage of methanol adaption (30~60th h) the fluctuation of dissolve oxygen was observed, and we gradually increased the air flow rate from 1 vvm to 3 vvm, to ensure the affordance of enough oxygen, and the DO was sustained at 20~15% during 60~144th h. After 144th h, air flow rate was adjusted back to 1 v.v.m. because of an increment of oxygen tension was observed.

After conducing ELISA for fusion protein assay and Bradford assay for total soluble protein quantification, the results showed that the fusion allergen appeared from 30th h. From 36th to 60th h, fusion allergen in medium increased rapidly and achieved a higher efficiency at 60th h. During 60th to 96th h, the fusion protein in medium dropped quickly and turned to slowly increase after 96th h. The concentration of fusion achieved another peak at 171st h, but the cultivation time was long and *Pichia pastoris* obviously began to die. The Western blotting also showed a similar trend to the ELISA results (Fig. 12).

Chapter IV

Discussion and Conclusion

4.1 Discussion

Mut^+ strains show metabolite methanol more efficient than Mut^s strains. *P. pastoris* X33 is a wild strain belongs to Mut^+ genotype, therefore the advantages of using X33 is mainly possessing a good growth rate when facing a methanol induction. In this study, the Mut^+ selection was conducted before Zoecin selections (for higher copy number strains) in order to avoid choosing a higher copy number but with a Mut^s genotype. During Mut^+ selections, 35 colonies were chosen from 50 colonies for growing well in MM plates and these strains were further screening in Zeocin-containing plates. Our results showed all strain grew well on plates with lower Zeocin concentrations (50 μg/mL and 100 μg/mL), but only few could grow on plates with higher Zeocin concentrations (500 μg/mL and 1000 μg/mL). It suggested that multiple recombination sites were happened after electroporation of vectors.

During Zeocin selections, 9 strains tolerant to 1000 μg/mL Zeocin were discovered (Fig. 3). After ELISA quantification of the fusion allergen, 3 strains showed higher productivity than 10 μg/mL after 24 h of methanol induction, and 5 strains showed the productivity lower than 10 μg/mL. Among these strains, No. 16 was found without the ability of fusion allergen expression. It suggests that strain 16 might be a Mut^s strain.

Our ELISA and Western blotting results indicated that the fusion allergen was secreted to the medium and the α-helix linker did work. Via a high cell density cultured in a fermentor (Fig. 9), we observed that before adaption to methanol, for *P. pastoris* methanol was used for induction of fusion allergen production and *P. pastoris* grew slowly. Once *P. pastoris* adapted to methanol, methanol would be used as the energy source to expand the biomass and secreted the *Pichia*-derived protease to decompose the expressed fusion allergen as the nutrients. When the biomass came into a saturation stage, the methanol was used for fusion allergen production again. In order to solve the disadvantage of co-expression of protease, *P. pastoris* strain such as SMD1168 and KM71 might be good choices. These strains are protease-deficient and therefore the recombinant proteins could be preserved during the methanol induction. In addition, to elongate the stage of glycerol-fed to achieve a higher biomass yield before methanol feeding is an alternative way.

The reports regarding productivity of Der p 1 or Der p 2 expressed in different hosts were summarized in Table 4. Reports show that crucial commitment of proteolytic activity of a *pichia*-derived rDer p1 to sensitization toward IgE and IgG responses (Takai et al., 2002; van Oort et al., 2004; Kikuchi et al., 2006). Der p 1 was first expressed in *E. coli* in 1988 however produced in the form of inclusion body that rDer p

1 was hardly to be determined. During 2000-2001, rDer p 1 was reported to be successfully expressed in *Drosophil*a cells (20 μg/mL) and mammalian cells (34 μg/mL) with a low productivity. Glycan test showed that N-glycosylation was not happened in natural Der p 1, but were observed in *Drosophil*a cells and mammalian cells (Jacquet et al., 2000; Massaer et al., 2001). Natural Der p 1 showed a sharp band on SDS-PAGE instead of a smear bands (Jacquet et al., 2002). In 2002, an expression of rDer p 1 in *P. pastoris* was reported and rDer p 1 in cultured medium was 70 μg/mL. However, the expressed rDer p 1 was found to be hyperglycosylated. Compared with the natural Der p 1, hyperglycosylation may lead rDer p 1 to be difficultly analyzed in vivo for losing its histamine release activity, proteolytic activity and the inhibition of IgE binding ability. After treated with N-Glycosidase, rDer p 1 recovered the similar activities to natural Der p 1 (Jacquet et al., 2002). N-Glycosidase therefore can be a way to remove glycosylation on the fusion allergen when we produce the rDer p 1 with a glycosylation. And another strategy was to take a site-direct mutation of proDer p 1 (N52Q) which optimized the rDer p 1 into a more similar character as the natural Der p 1 (van Oort et al., 2004).

In addition to the effect of glycosylation on its performance of protease activity, existence of the prosequence of natural Der p 1 is also thought to be an important issue.

Reports shows that rDer p 1 expressed with proDer p 1 domain design achieved a similar activity to natural Der p 1(Jacquet et al., 2000; Massaer et al., 2001). We realize that protease activity of Der p 1 was directly associated with the prodomain that would function as an intramolecular chaperon to help Der p 1 correctly folded into an active form, and it suggests that prosequence of Der p1 may be necessary for its immuno-efficacy in clinic trial. After the construction of full-length Der p 1 fused with Der p 2, this fusion allergen may have ability to facilitate itself to pass through epithelium cells and enhance the immune response. Besides the above reports, rDer p 1 had also already expressed in the rice as an edible vaccine and achieved a content of 50μg/grains.

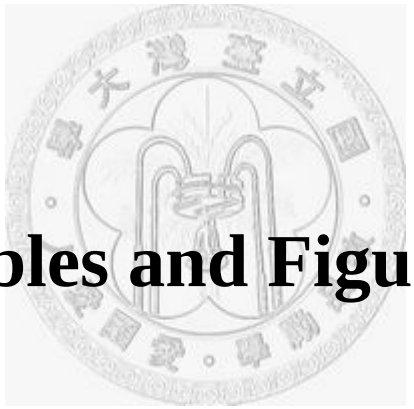
Der p 2 was also initially expressed in *E. coli* as an inclusion body. Expressed in eukaryotic system of *S. cerevisiae* (7 μg/mL) however was mostly to be degraded. Expression of rDer p 2 in tobacco showed a good activity in oral delivery in murine model of asthma (Ho, 2002) however with a low productivity. Co-expression of Der p 1 and 2 and in the form of a fusion allergen by *P. pastoris* is now first reported in this study. The productivity of fusion allergen achieved 203 μg/mL in 60 hours of the culture. This is also the highest yield among the related articles which had been reported.

In addition to the previous issues, switching the linker sequence into other linkers

with different characters is also an interesting work. Overall, to establish an economic, efficient, and to get an active form of fusion allergen, the choice of an adequate link is absolutely important.

4.2 Conclusion

In brief, we successfully linked the *Der p 1* and *Der p 2* genes with an α-helix forming nucleotide sequences. Via constructed in a pPICZαA vector, it was electroporated into *P. pastoris* wild strain. A high productivity strain (strain No. 33) for production of fusion allergen was chosen. The productivity of the fusion allergen in Hinton's flask was 35.5 μg/mL after 72 h of methanol induction. A high cell density culture in the Bioflo110 fermentor was achieved which caused to 203 mg/L fusion allergen productivity at 60 h and got a 427 g/L wet biomass at 132 h. This combination of the major allergens in *Dermatophagoides pteronyssinus* is suspected to be developed as an efficient vaccine for allergen disease treatment.



Tables and Figures

Table 3 House dust mite allergens. (Thomas et al., 2002)

Group	Biochemical function	MW cDNA[1] (SDS-PAGE)	Species[2]	IgE binding[3]
1	Cysteine protease	25,000	Dp, Df, Dm, Ds Em	80–100
2	Unknown (HE1 homologue)	14,000	Dp, Df, Ds, Em, Ld, Tp, Gd, As	80–100
3	Trypsin	25,000 (30,000)	Dp, Df, Ds, Em	16–100
4	α-Amylase	57,000	Dp, Em	40–46
5	Unknown	15,000	Dp, Bt, Ld	50–70
6	Chymotrypsin	25,000	Dp, Df	40
7	Unknown	25,000 (31,000, 29,000, 26,000)	Dp, Df, Ld	50
8	Glutathione-S-transferase	26,000	Dp	40
9	Collagenolytic serine protease	no cDNA, (30 000)	Dp	90
10	Tropomyosin	37,000	Dp, Df	50–95
11	Paramyosin	96,000 (92,000, 98,000)	Df, Bt	80
12	Unknown	14,000	Bt	50
13	Fatty acid-binding protein	15,000	Bt, Ld, As	10–23
14	Vitellogenin/apolipophorin-like	177,000 (variable)	Df, Dp, Em	90
15	98,000 Chitinase	62,500 (98,000, 105,000)	Df	70
16	Gelsolin	55	Df	35
17	Ca-binding EF protein	30	Df	35
18	Chitinase	60,000	Df	60
19	Anti-microbial peptide	7,000	Bt	10

[1] MW calculated from cDNA (SDS-PAGE of natural allergen, if different).

[2] Allergen described for the species designated by initials: *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Euroglyphus maynei, Dermatophagoides siboney, Dermatophagoides microceaus, Lepidoglyphus destructor, Blomia tropicalis, Tyrophagus putrescentiae, Glycophagus domesticus, Ascaris siro.*

[3] Binding frequency (% patients, variation due to patient selection).

Table 2 Recombinant allergens expressed in various systems. (Schmidt and Hoffman, 2002)

Expressed in	Allergen	Comments
E. coli	Api m 1	Renatured to full enzyme and IgE-binding activities
	Api m 2	Only 20–30% enzyme activity after renaturation
	Cyp c 1	Produced in fully active form when calcium added
	Ara h 1	Codon usage requires specially engineered strain
	Bet v 1	Isoforms with varying IgE-binding activity
	Cor a 1	Bet v 1 cross-reactive, isoforms
	Bla g 4	Low yield
	Fel d 1	Not glycosylated
	Ves v 5	Insoluble
	Cyn d 1	No IgE-binding activity
Pichia	Cyn d 1	IgE-reactive
	Alt a 1	IgE-reactive, glycosylated
	Mus m 1	Native conformation
	Bla g 4	High yield
	Fel d 1	Glycosylated
	Der p 1	Hyperglycosylated precursor
	Ves v 5	High yield, native conformation
Saccharomyces	Der p 1	Insoluble
	Der p 2	IgE-binding activity
	Procalin	Immunoreactive
Baculovirus	Api m 2	Full enzymatic and IgE-binding activities
	Sol i 2	IgE-reactive, native conformation, natural cleavage of initiation sequence
	Fel d 1	Fully immunoreactive
	Lep d 2	Isoforms produced
	Mal f 1	Similar to that expressed in *E. coli*
	Cte f 1	Fully reactive with IgE antibodies
	Aed a 1	Immunoreactive apyrase
Mammalian	Per a 1	Low yield
Plant	Bet v 1	IgE binding, mouse antibody binding
	Hev b 1	IgE binding
	Hev b 3	IgE binding

Table 3 Primer design and the linker sequence.

Fragment	nucleotides sequence
pEcoRI-Der p 1	5'-AAG-GAATTC-ACT AAC GCC TGC AGT ATC AA-3'
pDer p 1-XhoI	5'-CTG-CTCGAG-GAG AAT GAC AAC ATA TGG AT-3'
pXhoI-linker	5'-ACA-CTCGAG-GGT TCT ACC TCT-3
plinker-SacII	5'-TTA-CCGCGG-GAT ACC AGA ACC-3'
pSacII-Der p 2	5'-AAT-CCGCGG-GAT CAA GTC GAT GTC AAA GA-3'
pDer p 2-XbaI	5'-ACA-TCTAGA-CC-ATC GCG GAT TTT AGC ATG AGT-3'
Linker sequence	5'-ACA-CTCGAG-GGT TCT ACC TCT GGT GGT TCT ACC TCT GGT GGT TCT ACC TCT GGT TCT GGT TCT GGT ATC-CCGCGG-TAA-3'

Table 4 Comparisons of rDer p 1 and rDer p 2 expressed in different hosts

allergen	system	concentration	year
Der p 1	*E. coli*	low	1988
Der p 1	*Drosophila* cells	20 μg/mL	2000
Der p 1	mammalian cells	34 μg/mL	2001
Der p 1	*Pichia pastoris*	70 μg/mL	2002
Der p 1	rice	50μg/grains	2008
Der p 2	*E. coli*	50 μg/mL	1997
Der p 2	*Saccharomyces cerevisiae*	7 μg/mL	1998
Der p 2	*Tobacco*	8 μg/mL	2002
Der p 1+Der p 2	*Pichia pastoris*	up to 203 μg/mL	2008

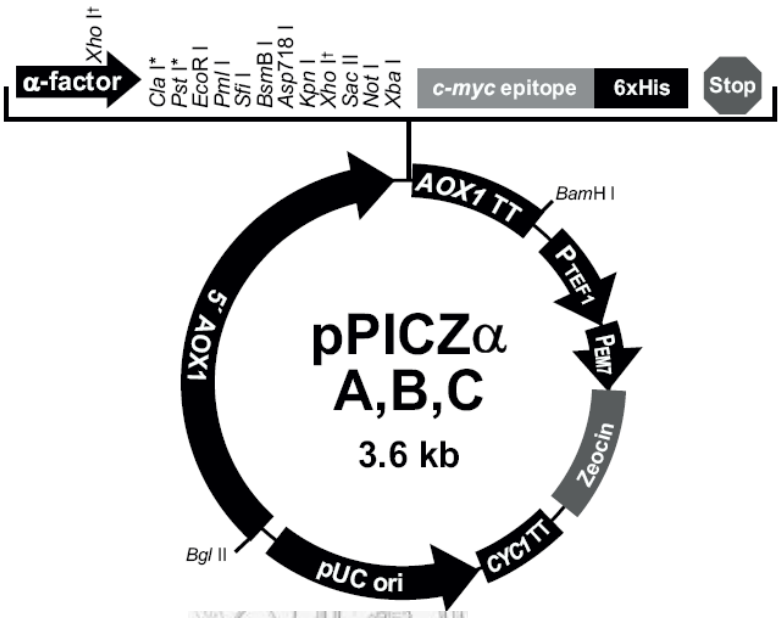


Figure 1 pPICZαA vector. (A) is the multiple cloning site of pPICZαA and (B) is the vector map. The figure and the complete sequence of pPICZαA is available for downloading from Invitrogen company's World Wide Web site (www.invitrogen.com).

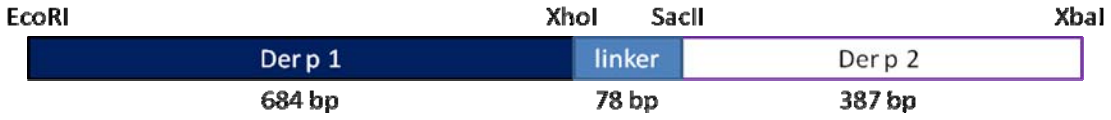


Figure 2 Schematic representation of Der p 1 and 2 fusion allergen.

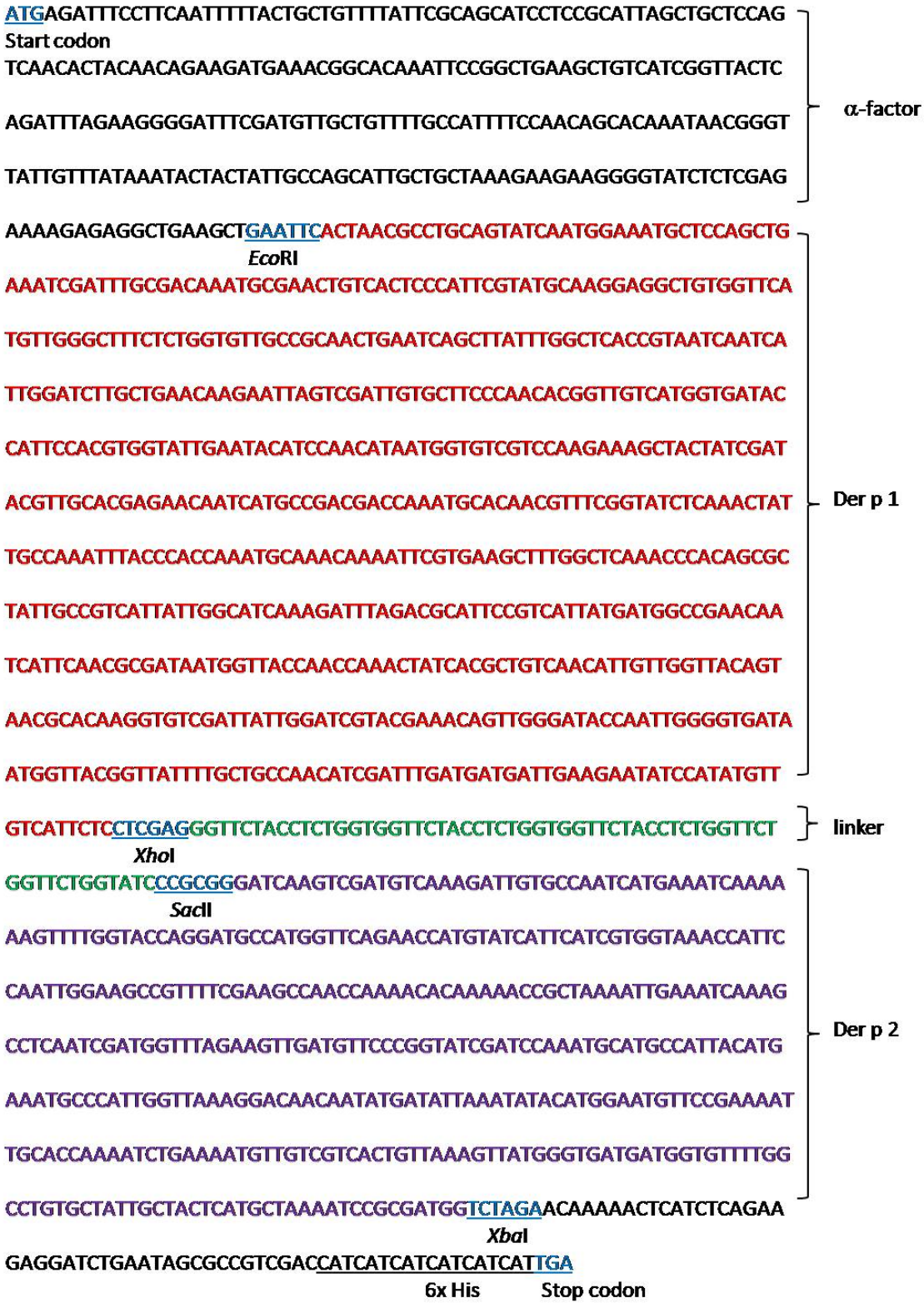


Figure 3 Sequencing result of fusion gene.

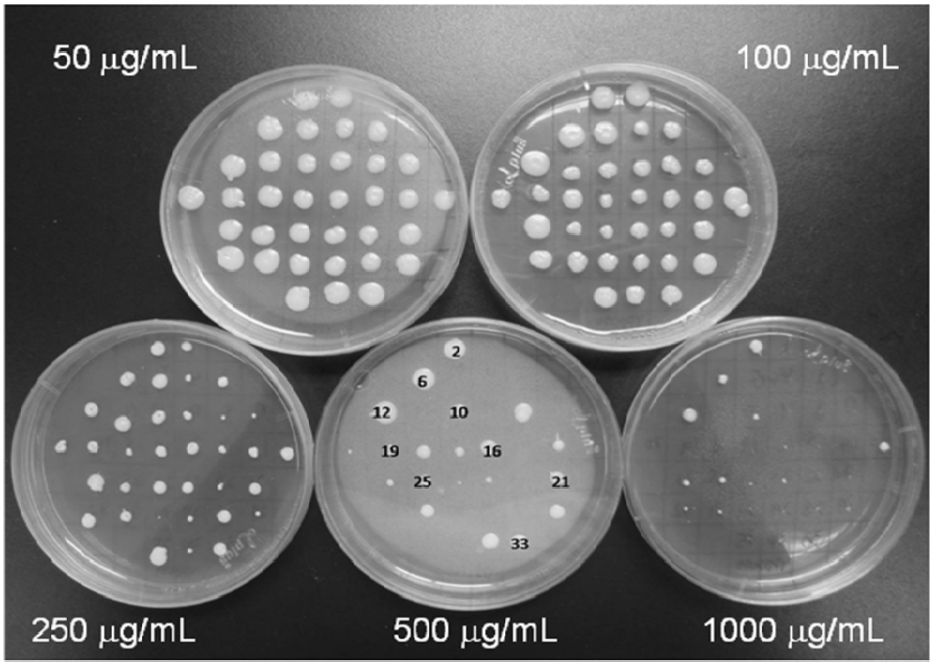
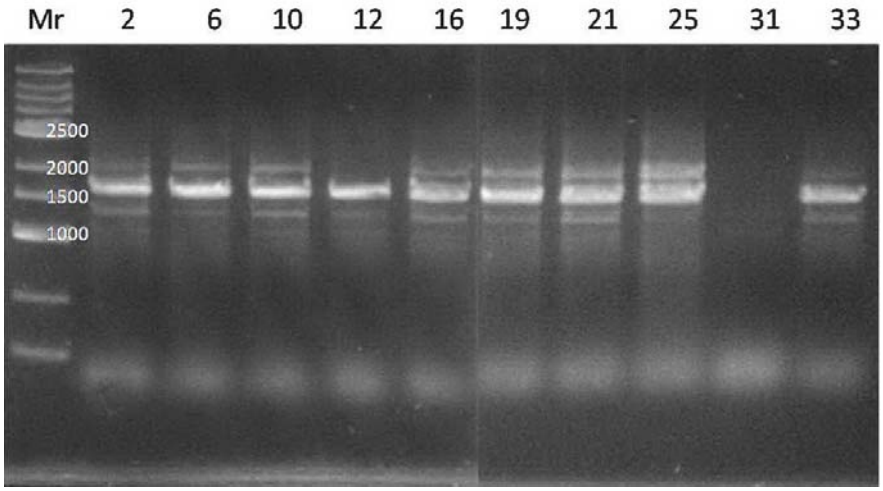
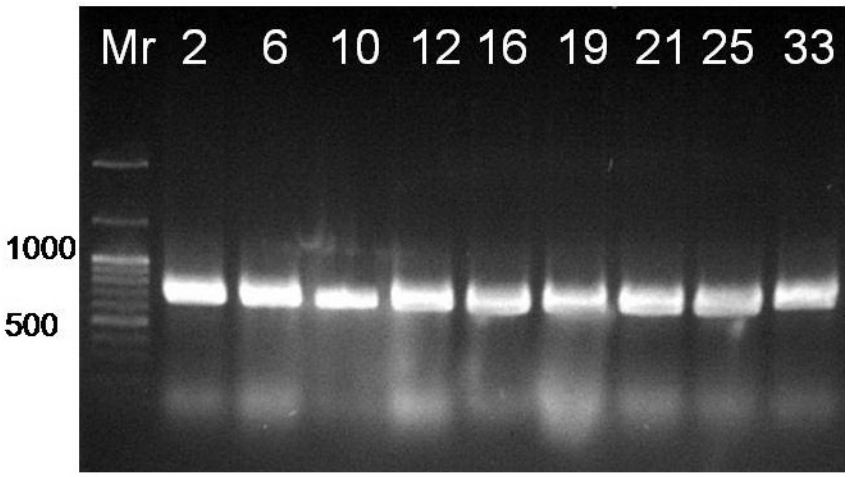


Figure 4 Results of the Zeocin selection. Thirty five strains cultured on YPDZ plate for 4 days and the Zeocin concentrate was from 50 μg/mL to 1000 μg/mL.

5-1



5-2



5-3

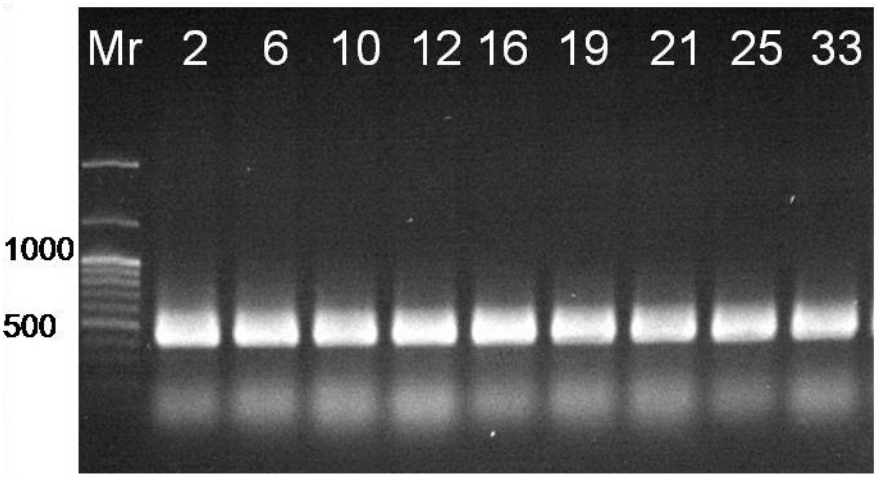


Figure 5 Colony PCR result. 5-1, AOX primer. 5-2, Der p 1+linker primer. 5-3, linker+Der p 2 primer

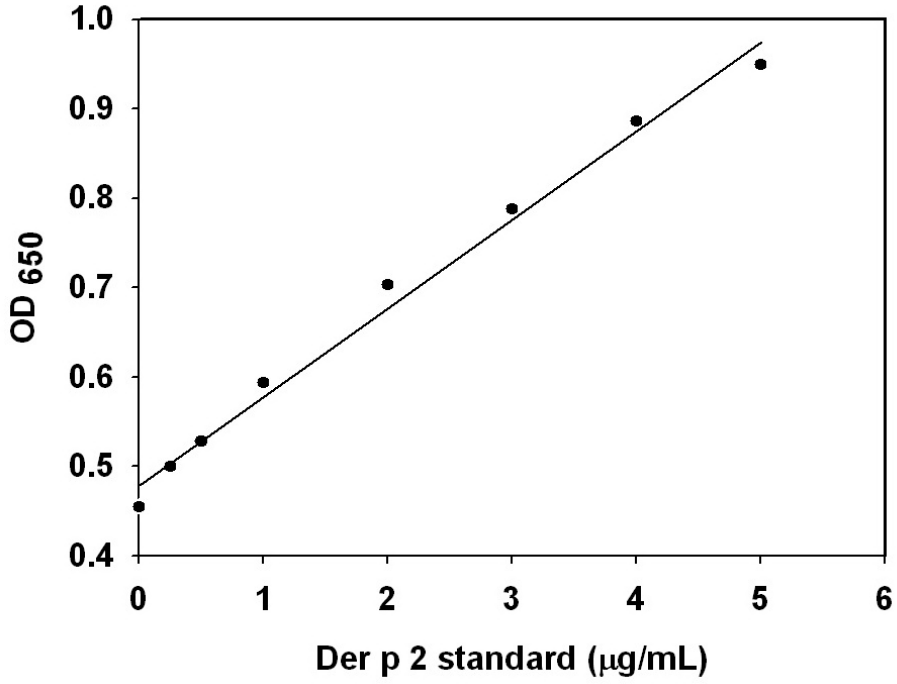


Figure 6 Standard curve of Der p 2 ELISA.

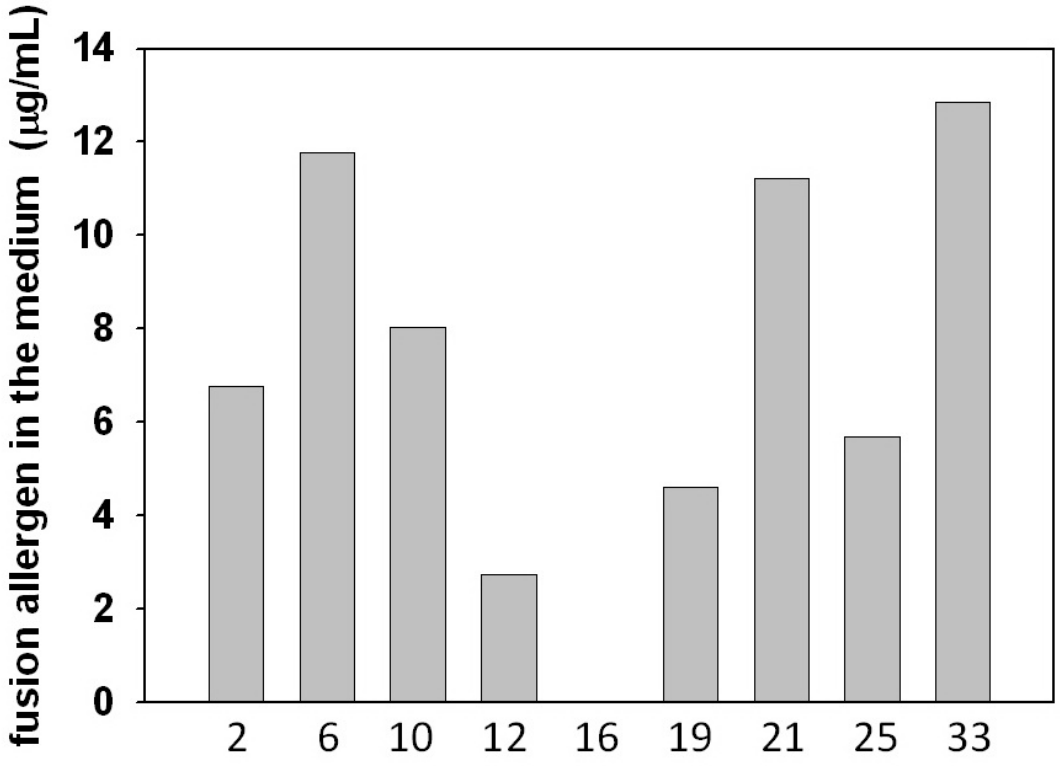


Figure 7 Selection results of 9 strains cultured in a small scale methanol induction.

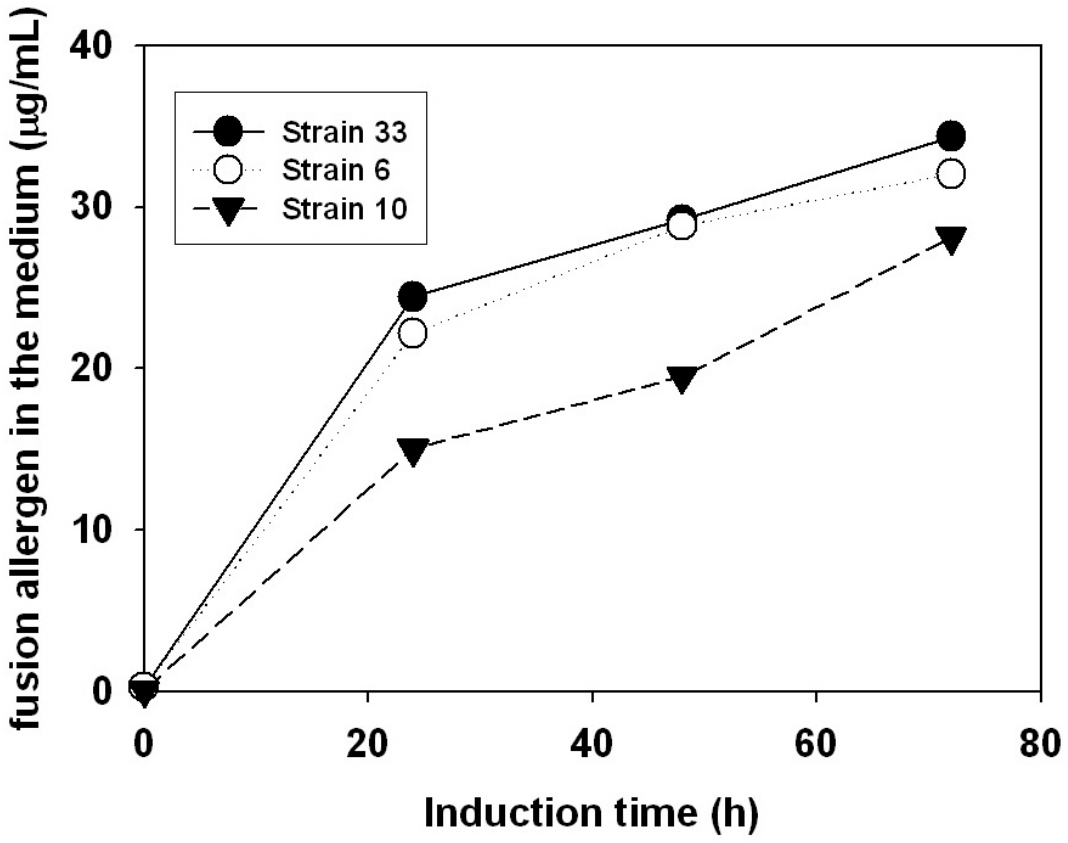


Figure 8 Fusion allergen expression efficiency of strain 6, 10 and 33.

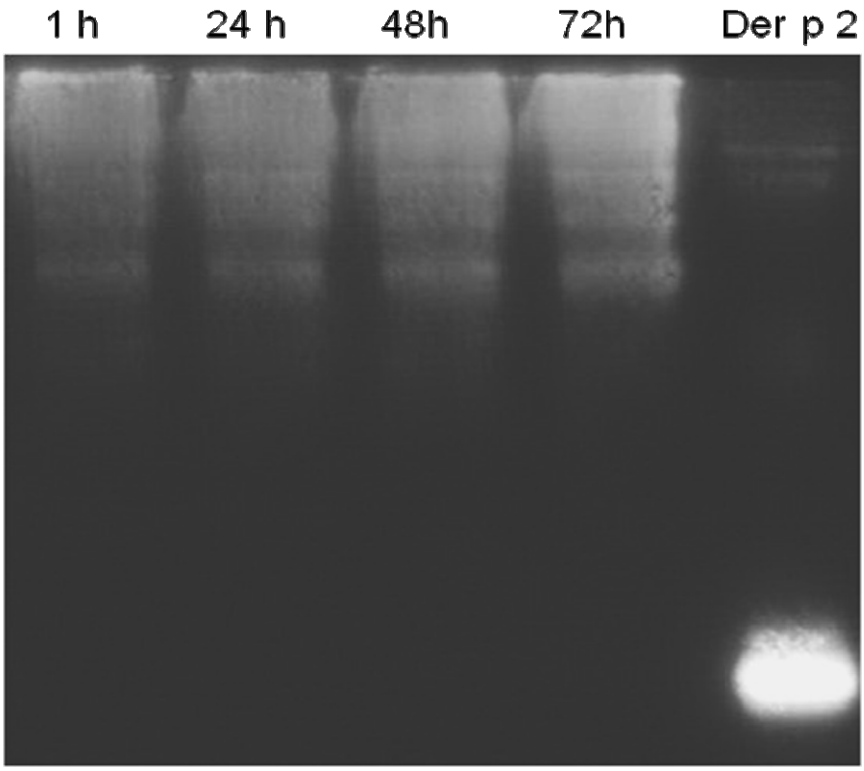


Figure 9 Western blotting results of strain 33 cultured in Hinton's flasks.

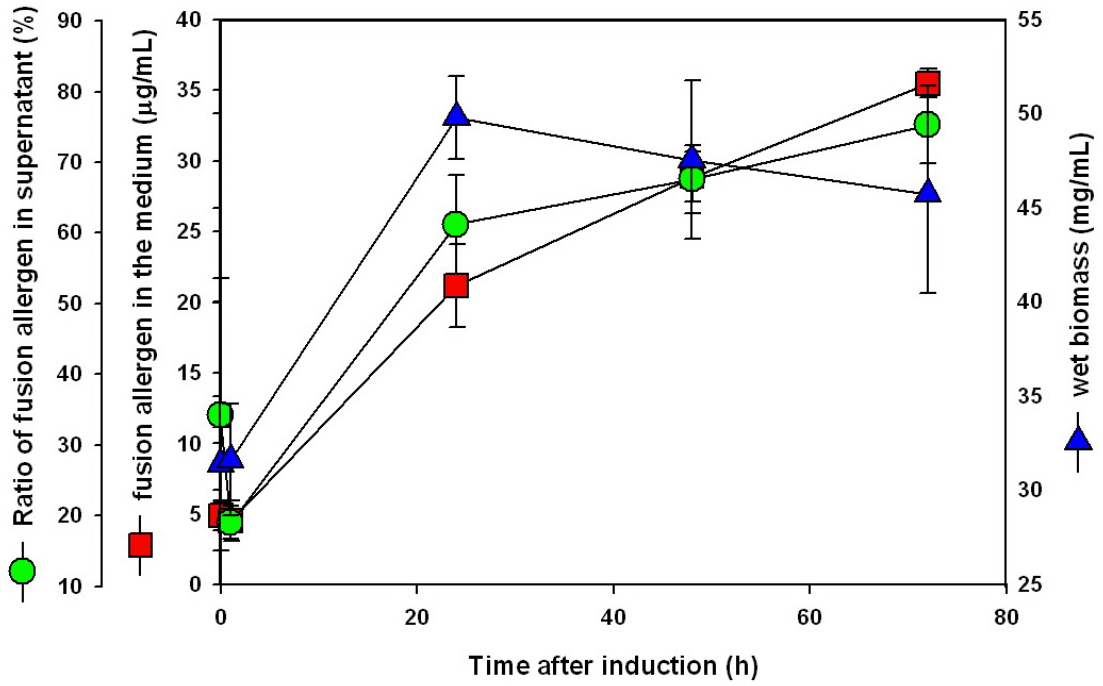


Figure 10 Strain 33 cultured in Hinton's flasks.

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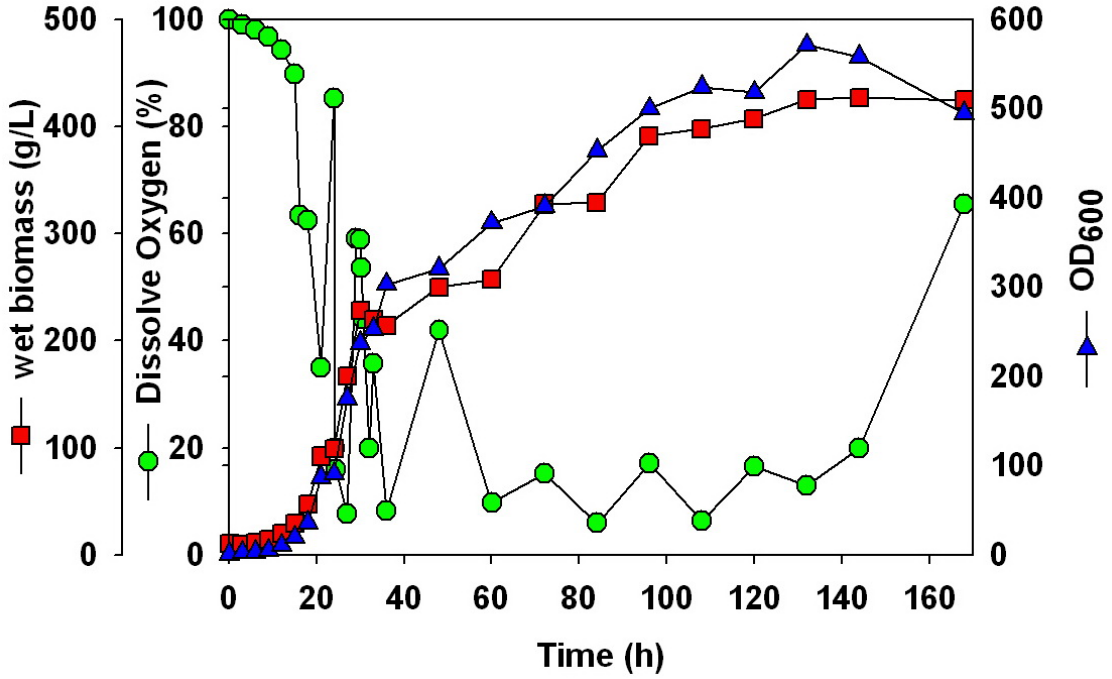

Figure 11 Strain 33 cultured in a fermentor.

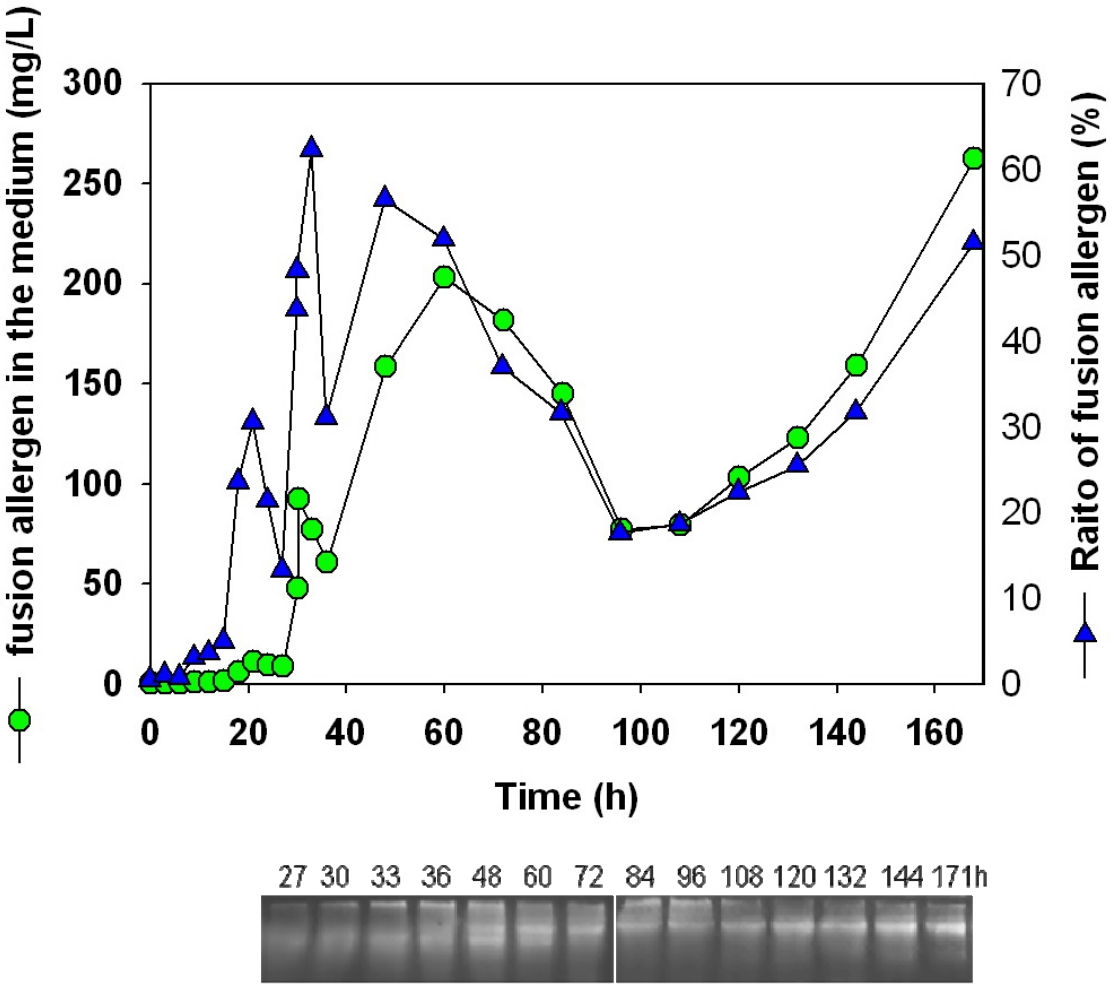

Figure 12 ELISA and the Western results of strain 33 cultured in a fermentor.

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Appendix

Media for yeast:

Yeast Extract Peptone Dextrose broth (YPD, per liter)

Yeast Extract	10 g
Peptone	20 g
Glucose	20 g

Yeast Extract Peptone Dextrose agar (YPD, per liter)

Yeast Extract	10 g
Peptone	20 g
Glucose	20 g
Agar	20 g

Yeast Extract Peptone Dextrose agar with Zeocin (YPDZ, per liter)

Yeast Extract	10 g
Peptone	20 g
Glucose	20 g
Agar	20 g

*autoclaved and cool solution to below 60^{o}C before antibiotics were added

Zeocin (100 mg/ mL stock)	1 mL

Increase the usage of Zeocin stock to formulate different concentration of Zeocin

YPD agar with sorbitol and Zeocin (YPDSZ, per liter)

Yeast Extract	10 g
Peptone	20 g
Sorbitol	182.2 g
Agar	20 g

* add water to 900 mL autoclaved and cool solution to below 60^{o}C

Glucose (20%)	100 mL
Zeocin (100 mg/ mL stock)	1 mL

BMG broth	**(per liter)**
Potassium phosphate	100 mM
	* adjust pH to 6.0
Yeast Nitrogen Base	13.4 g
d-biotin	0.4 mg
glycerol	10 mL
	* the above 3 were filter steriled by 0.22 mm filter membrane

BMM broth	**(per liter)**
Potassium phosphate	100 mM
	* adjust pH to 6.0
Yeast Nitrogen Base	13.4 g
d-biotin	0.4 mg
methanol	5 mL
	* the above 3 were filter steriled by 0.22 mm filter membrane

Media for *E. coli*

Luria-Bertani agar	**(LB, per liter)**
Tryptone	10 g
Sodium Chloride	10 g
Yeast Extract	5 g
	* adjust pH to 7.5
Agar	15 g

LB broth with ampicillin	**(LBA, per liter)**
Tryptone	10 g
Sodium Chloride	10 g
Yeast Extract	5 g
* adjust pH to 7.5, autoclaved and cool solution to below 60^{o}C before antibiotics were added	
Ampicillin (50 mg/mL stock)	2 mL

LB agar with ampicillin	**(LBA, per liter)**
Tryptone	10 g
Sodium Chloride	10 g
Yeast Extract	5 g
	* adjust pH to 7.5
Agar	15 g
	*autoclaved and cool solution to below 60°C before antibiotics were added
Ampicillin (50 mg/mL stock)	2 mL

Low salt LB Agar with Zeocin	**(LSLBZ, per liter)**
Tryptone	10 g
Sodium Chloride	5 g
Yeast Extract	5 g
	* adjust pH to 7.5
Agar	15 g
	*autoclaved and cool solution to below 60°C before antibiotics were added
Zeocin (100 mg/mL stock)	250 μL

Buffers and solutions

50X TAE buffer	**(per liter)**
Tris Base	242 g
Acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
	* 1X TAE buffer is obtained by diluting in ddH_2O 50 times

A solution	**(per 50 mL)**
Acrylamile	14.6 g
Bis	0.4 g

* store at 4°C

B solution	**(per 100 mL)**
Tris	18.2 g
TEMED	0.36 mL

* adjust pH to 8.8, store at 4°C

C solution	**(per 100 mL)**
Tris	0.6 g
TEMED	0.04 mL

* adjust pH to 6.8, store at 4°C

2X SDS sample buffer	**(per 10 mL)**
Tris Base	0.3 g
SDS	0.4 g
EDTA · 2Na	14.9 g
β-mercaptoethanol	1 mL

Adjust pH to 6.8

10% Ammonium persulfate	**(APS, per mL)**

Dissolve 0.1 g into 1 mL H_2O, perpare on the day used

Tracking dye	**(per 10 mL)**
Bromophenol blue	1 mg
1X TBE	5 mL
Glycerol	5 mL

Plasmid construction

PCR

Super-Therm DNA Polymerase (Bertec, Taipei, Taiwan)

dNTP mixture (Viogene, CA, USA)
10x PCR reaction buffer (Bertec, Taipei, Taiwan)

Restriction enzyme
*Eco*RI (Takara, Shiga, Japan)
*Xba*I (Takara, Shiga, Japan)
*Xho*I (Takara, Shiga, Japan)
*Sac*I (Takara, Shiga, Japan)
*Sac*II (Takara, Shiga, Japan)

yT&A cloning vector kit (Yeastern Biotech, Taipei, Taiwan)
T4 DNA ligase (Takara, Shiga, Japan)
QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany)
Gel extraction kit (Geneaid, Taipei, Taiwan)
T4 DNA ligase (Takara, Shiga, Japan)

Protein purification and detection

Protein quantity
Bradford (Bio-Rad, CA, USA)

Western blotting
Mouse anti-Der p 2 antibody generated hybridorma
(kindly provided by Professor Chiang)
Or Rabbit anti-Der p 2 antibody made by Genesis Biotech Inc.
Western lightning-Chemical Reagent Plus (PerkinElmer Life Science, Inc., MA, USA)
Hybond-C Extra membrane (Amersham, London, UK)
Goat anti-Rabbit IgG (H+L) HRP conjugated (Chemicon, CA, USA)
Transfer buffer: 192 mM Glycine, 25mM Tris, 20% Methanol
10x TBS: 0.5M Tris Base, 9% NaCl, pH 7.6
Blocking buffer: 1X TBS, 5% skim milk
Wash buffer (TBST): 1X TBS, 0.25 % Tween20

ELISA
Mouse anti-Der p 2 antibody
Rabbit anti-Der p 2 antibody made by Genesis Biotech Inc.

Goat anti-Rabbit IgG (H+L) HRP conjugated (Chemicon, CA, USA)

1-Step^{TM} Ultra TMB-ELISA (Pierce, IL, USA)

Coating buffer: carbonate-bicarbonate buffer (0.015 M Na_2CO_3, 0.03 M $NaHCO_3$), pH 9.6

PBS: 0.02 M sodium phosphate buffer with 0.15 M sodium chloride, pH 7.4.

Wash buffer (PBST): 1X PBS, 0.05 % Tween20

Gelatin-NET: 0.25% gelatin, 0.15 M NaCl, 5 mM EDTA, 0.05% Tween in 50 mM Tris-HCl, pH 8.0